PATENT
Attorney Docket No. 15422-70

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	
Russell G. Higuchi, et al.	Examiner: R. Prouty
Serial No.: 07/695,201	Art Unit: 1814
Filed: May 2, 1991	DECLARATION PURSUANT TO 37 CFR § 1.132
For: HOMOGENEOUS METHODS FOR NUCLEIC ACID AMPLIFICATION AND DETECTION	

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Dr. Russell G. Higuchi, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true and statements made on information or belief are believed to be true. The Exhibits (1-6) attached hereto are incorporated herein by reference.

2. I received a Ph.D. in molecular biology from the University of California at Los Angeles in 1980. A copy of my curriculum vitae is attached as Exhibit 1.

3. I am presently employed by Roche Molecular Systems as a Research Investigator. My primary responsibilities include directing and conducting research to improve PCR and find new applications for PCR.

4. I have personally reviewed the subject application and the Patent Examiner's rejection of the pending claims. The purpose of this declaration is to (1) explain that Sutherland et al., U.S. Patent No. 5,049,490, does not describe PCR; (2) explain that modification of the reference of Sutherland to embrace PCR would render the method so complicated that it would be useless for the proposed purpose set forth by Sutherland; (3) explain that to modify the PCR of Mullis using the methods of Sutherland would run counter to the purpose of Mullis; and (4) describe the surprising and unexpected results of my invention.

5. **The Sutherland Patent Does Not Describe PCR.**

I have reviewed the Sutherland patent and am familiar with its contents. Example 6 has been asserted against the subject claims because of its recitation at column 16, lines 60-63. The text is as follows:

> The polymerase chain reaction was then initiated by the addition of aliquotes of DNA polymerase, and fluorescent signals were measured continuously over time.

This passage recites an error which is obvious to scientists familiar with PCR. It is an oversight or sloppy use of scientific language.

PCR is an amplification procedure which requires an excess amount of two primers and repeated thermal cycling to permit annealing and denaturing of primers and extension product. The Sutherland patent is directed to measuring the amount of *polymerase* present in a sample. In the process of developing commercial kits, Cetus\Kodak (the assignees of the Sutherland patent) were interested in better ways of measuring the amount and quality of polymerase present in our PCR

products. The reagents used in Example 6 are PCR reagents. PCR reagents were used, not because the investigators intended to measure amplification target during PCR, but because they wanted measure the amount of polymerase in PCR mixtures. The passage relied upon by the Examiner and cited above is clearly an error in language. There is no PCR described in that passage nor anywhere else in the application. It is beyond question that the recited reaction mixture of Example 6 has only one primer and the assay described does not involve thermal cycling.

6. I further understand that the Examiner finds that the Sutherland/Mullis disclosures would motivate one of skill to combine agents whose fluorescence is enhanced upon binding duplex DNA with PCR, not merely PCR reagents. Declarant respectfully disagrees. First, it will be explained that one of skill reading Sutherland would not be motivated to modify the reactions there described with the PCR of Mullis. Then it will be explained why one of skill would not be motivated to modify Mullis by adding the DNA binding agents of Sutherland.

7. **There Is No Motivation To Use PCR To Quantify DNA Polymerase.**

With regard to modifying Sutherland by the teachings of Mullis, this would greatly complicate the polymerase assay and would decrease its accuracy if not render it inoperable. The quantification of polymerase using the Sutherland method relies on the consistent and predictable rate of nucleotide extension provided by the polymerase enzyme. The proposed modification would involve substituting the single primer/single-step/single-temperature extension reaction of Sutherland with the two primer/multi-step/multi-temperature thermal cycling reaction of PCR. This use of PCR complicates the Sutherland assay in at least three distinct parameters. Each effected parameter is altered in an unpredictable manner and each individually casts doubt upon the quantitative capabilities of the hypothetical PCR/Sutherland polymerase assay.

First, PCR uses repeated heating and cooling steps in the reaction. Since, in an enzyme catalyzed reaction, the rate is dependent on temperature, the changing temperatures in a PCR would complicate measurement of reaction rate so severely that accurate and reproducible polymerase quantitation would be impossible. Consequently, the combination of PCR and the assay described in the Sutherland et al. reference would render that assay useless for the purpose and calculation described in the reference. In contrast, fluctuations in reaction rate are not problematic in a PCR because the critical factor in PCR is the accumulation of PCR product, not enzyme quantitation based on activity.

Second, PCR uses two primers binding to different nucleic acid sequences. In addition, the base sequences downstream from the two primers are different. For these reasons, it could be stated that PCR involves two separate extension reactions proceeding independent of each other. Because Sutherland relies upon predictable rates of polymerase 3' extension, introduction of a second and simultaneous extension reaction will force the user to quantitate an average between the two different reactions. This two reaction average will be intuitively less accurate than a system relying on a single reaction. There is simply no motivation to complicate and reduce the accuracy of the quantitation method of Sutherland by introducing a second independent reaction.

Third, the introduction of a DNA binding agent will effect the melting of the nucleic acid duplex. This is not a problem in Sutherland which utilizes a single-stranded DNA template, but the effect upon melting will inevitably impact on PCR efficiency, and thus upon an assay measuring polymerase activity through PCR.

We have evaluated the accumulation of PCR product in the presence of EtBr varying only polymerase quantity. The results were as predicted and support the fact that PCR is a poor substitution of the single-step systems of Sutherland. More specifically, the Sutherland assay has demonstrated utility from 0 to 30 units of polymerase. (See patent Figs. 1, 5, 9, and 10) In contrast, the use of PCR

narrowed the range to a single unit rendering the method useless for all practical matters.

Exhibit 2 graphically demonstrates the unsuitability of PCR conditions for quantifying polymerase activity. The exhibit provides a graph showing fluorescence due to the accumulation of double-stranded DNA in PCRs containing EtBr, at each cycle, with varying amounts of enzyme. The reactions were carried out in accordance with my invention as described in the examples of the above-referenced patent application. Only polymerase concentration was varied.

Because PCR is an exponential reaction, the yield of double-stranded DNA is not simply related to enzyme concentration. At <1 unit of enzyme there is no detectable double-stranded DNA produced. Thus, for quantitating the amount of enzyme present, where enzyme concentration is unknown, the Sutherland et al. method combined with PCR would fail to accurately determine enzyme activity where less than one unit of enzyme is present.

Exhibit 2 further demonstrates that the Sutherland et al./homogeneous PCR assay envisioned by the Examiner also fails for quantitating concentrations of enzyme higher than approximately 2 units of enzyme. In PCR the accumulation of product can be limited by reagents other than enzyme, e.g., primers, such that the rate of product formation eventually reaches a plateau. Therefore, quantitation of enzyme activity becomes increasingly inaccurate as the amount of enzyme is increased. Having demonstrated the inability of PCR to quantitate polymerase concentration over a useful range, the Examiner should note that in Figures 5, 9, and 10 of Sutherland, their assay functions well from 0 to 30 units. Thus, I submit that as a "Sutherland et al. type" assay, the modified assay provided by the use of PCR provides an inaccurate and undesirable assay of no practical value.

In addition, applying the conditions of Mullis et al. to Sutherland et al. renders the assay much more sensitive to the effect of enzyme inhibitors. This is illustrated in Exhibit 3. The experiment shown in the exhibit was conducted as

described in the present patent application, with the exception that hematin, a non-fluorescent PCR inhibitor, was included in varying concentrations. The sensitivity of PCR to inhibitors is clearly demonstrated in the exhibit. The slight inhibition due to 0.1 uM hematin severely depresses PCR. However, in contrast to PCR, in a Sutherland et al.-type assay, partially inhibitory effects do not affect enzyme quantitation because the method requires that fluorescence is compared to a base line measurement, subject to the same inhibitory effect. Clearly, the "guidance" provided by the Sutherland et. al reference is not suitable for combination with PCR.

8. **There Is No Motivation To Modify Mullis Using Sutherland.**

Having explained that there is no reason to modify Sutherland with Mullis, it is also true that there is no motivation to modify Mullis using Sutherland. In contrast to Sutherland which discloses a single step method for quantifying polymerase amount in a PCR reaction mixture and one primer, Mullis discloses a multi-step method of amplifying a target nucleic acid using two primers. Mullis further motivates those of skill to quantify the amount of amplification product at the completion of the process.

As I understand the basis for the rejection - the examiner is concerned that the Sutherland discussion of DNA binding agents in a PCR reaction mixture would motivate, one to conduct PCR using DNA binding agents. This is not true.

Mullis is premised on a motivation to obtain large quantities of nucleic acid using a method that exponentially increases copies of DNA. DNA binding agents are known to inhibit polymerase activity and even a slight inhibition of polymerase can deleteriously affect the ability of PCR to produce nucleic acid. Since Sutherland et al. measures polymerase activity against a standard of known activity under the same conditions, even substantial inhibition by DNA binding agents could remain undetected. For this reason no ordinary artisan would have

been motivated to introduce Sutherland's DNA binding agents into the PCR of Mullis. Furthermore, because Mullis is teaching amplification of nucleic acid, the only purpose of the adding DNA binding agents to PCR would be to measure amplification product at the end of thermocycling. For this purpose, such addition could be made after PCR is complete; there is no need to have DNA binding agents present during amplification. In contrast, my invention uses the addition of such reagents during PCR to obtain quantitative information about the starting amount of DNA and information about the efficiency of PCR under various conditions.

Having explained that the only reason to add DNA binding agents to a PCR would be to quantify amplification product, one needs to evaluate whether the prior art references would motivate one to add DNA binding agents to a PCR assay before amplification is complete. The answer is a clear no. Prior to my invention, quantitation of PCR product was carried out at the end of a selected thermocycle. Various comparisons are then made. It would be counter-productive to measure end product by prematurely adding a polymerase inhibitor which would have unknown inhibitory effects on the quantity of amplification product.

To illustrate the potentially dramatic effect of a slight inhibition of polymerase activity on the quantity of amplification product, I have attached a graphic representation of the impact of relatively low decreases in efficiency of DNA replication on the amount of product produced. The graphic representation is Exhibit 4 which is attached hereto. The Examiner should note that the average number of thermocycles for PCR is between 25 and 40. At 37 cycles, the decrease in final product at a 5% reduction in amplification efficiency is over 50%. With a 10% reduction in DNA replication efficiency, the amount of PCR product is decreased by 90% from the amount expected in the absence of inhibition.

Exhibit 2 also illustrates this effect. A very low concentration, i.e., 0.1 μM, of an inhibitor, hematin, reduces the yield of product by one half and delays the appearance of a detectable level of DNA product by many cycles. Using only 0.2

μM hematin, no detectable DNA product is ever made. The concentrations of fluorescent DNA binding agents used in my invention are ~100 times this level. Prior to my invention it was not obvious that such concentrations would allow PCR. Similarly, simply because a substance shows no effect on DNA polymerase activity, it does not mean that the substance will not adversely affect PCR. It is known, for example, that 1 M urea 10% formamide, and 5-10% n-methyl-pyrolidone all have a stimulatory effect on Taq DNA polymerase activity as measured by a Sutherland-type assay. However, at these concentrations all these reagents prevent PCR amplification. It would therefore be incorrect to conclude that the dyes used or proposed for use in Sutherland et al. would also permit PCR amplification.

9. **The Use Of DNA Binding Agents Provides A Surprisingly Advantageous Assay.**

Since Mullis invented the amplification of nucleic acid, well known alternatives for assaying amplification products of PCR have been developed. For example, one can use electrophoresis technology such as agarose electrophoresis and ethidium bromide as a stain. Such methods are quantitative as well as qualitative, but are confined to measuring end point levels of amplification product.

In contrast to measuring PCR product at the end of each cycle, our invention determined that the inhibition of PCR by DNA binding agents at the recited concentrations was surprisingly low and that the yield of the amplification product was not significantly affected by the presence of agents whose fluorescence is enhanced upon binding double-stranded DNA. This discovery gave rise to the invention of using the DNA binding agents in commercial PCR. Such applications require reproducible and consistent results.

The addition of DNA binding agents also permits real time monitoring of amplifications for the appearance of amplification product. In performing such

monitoring, it was apparent that the fewer cycles it takes for detectable PCR product to appear, the higher was the initial amount of DNA template. This observation is the basis of this invention's quantitative assay for DNA concentration.

Exhibit 5, which is attached hereto, demonstrates that by real-time continuous monitoring of a PCR, by the methods of my invention, for any amount of target present in the original sample, the cycle number at which PCR product becomes detectable is readily apparent. By determining the number of cycles needed to reach a fixed amount of fluorescence, e.g. a reading of 190, the claimed method provides that the cycle number is directly proportional to the logarithm of target copy number in the original sample. This linear, proportional relationship is shown in Exhibit 6, which is attached hereto.

The advantages of such an arrangement, over the measurement of PCR product after completion of a cycle, are apparent. All PCR amplifications eventually reach a "plateau" in which additional cycles add little PCR product. This plateau level of product may be the same no matter the starting level of target DNA. Because of this, when comparing two PCRs that are both in plateau, the amount of end product may provide little information about initial template DNA concentration. This is why measuring end points often precludes accurate estimates of starting copy. The prior art quantification methods had severely limited dynamic range. The term "dynamic range" refers to the range of starting a DNA concentration that can be accurately quantitated. Even when end-product levels of PCR product could reflect initial template DNA concentrations, small variations in the measurement of end product could lead to widely varying estimates of those concentrations.

My claimed invention avoids these pitfalls by continuously monitoring PCR product levels, using the fluorescence of the added DNA binding agents, and determining the cycle number at which a PCR reaches a level of fluorescence that

is independent of the plateau level. As shown in Exhibit 6, these measurements of initial DNA levels are relatively precise and the dynamic range of such measurements is quite broad. Thus, by practice of the present invention the range of starting DNA concentration that can be accurately quantitated is extraordinarily broad, spanning over 6 orders of magnitude.

Furthermore, these measurements are made without having to open the reaction vessel to further process the sample. This aspect of my invention saves labor, saves time, saves materials and greatly diminishes the opportunity for cross contamination, which is a critical issue in accessing the reliability and reproducibility of PCR data.

The availability of a broad dynamic range for quantitating target in a biological sample represents a tremendous advance over prior methods that require either a dilution series or other titration to extend their range. These advantages are particularly critical where the amount of sample is limited such as in forensic sciences or criminology where only trace evidence exists, or where vast numbers of sample are to be analyzed such as in a clinical setting. A broad range of accurate quantitation is essential for reliable AIDS virus detection and monitoring the patient response to, for example, experimental drug therapy where the result are non-predictable.

Finally, this invention is an advance over previous methods by allowing one to follow the progress of a given amplification. First, this is particularly useful when developing commercial PCR assays. When developing commercial assays, it is important to determine the effect of varying amplification conditions such as enzyme level on the test results. Real time quantification permits measurements of assay efficiency not previously possible. Second, when using the claimed assay in a commercial setting, this invention allows one to detect the presence of PCR inhibitors as is shown in Exhibit 2. Inhibitors are frequently found to contaminate samples from a clinical or forensic source, and their presence would severely

compromise the quantitation of initial DNA concentration. Knowing that an inhibitor is present allows the diagnostician to take steps to remove it from the sample to assure that subsequent results are reliable .

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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5/21/93
Date